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Molecular cloning and expression of two cDNAs encoding asparagine synthetase in soybean

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Abstract

Two cDNA clones (SAS1 and SAS2) encoding different isoforms of asparagine synthetase (AS; EC 6.3.5.4) were isolated. Their DNA sequences were determined and compared. The amino-terminal residues of the predicted SAS1 and SAS2 proteins were identical to those of the glutamine binding domain of AS from pea, asparagus, *Arabidopsis* and human, suggesting that SAS1 and SAS2 cDNAs encode the glutamine-dependent form of AS. The open reading frames of SAS1 and SAS2 encode a protein of 579 and 581 amino acids with predicted molecular weights of 65 182 and 65 608 Da respectively. Similarity of the deduced amino acid sequences of SAS1 and SAS2 with other known AS sequences were 92% and 93% for pea AS1; 91% and 96% for pea AS2; 88% and 91% for asparagus; 88% and 90.5% for *Arabidopsis*; 70.5% and 72.5% for *E. coli asn*B and 61% and 63% for man. A plasmid, pSAS2E, was constructed to express the soybean AS protein in *Escherichia coli*. Complementation experiments revealed that the soybean AS protein was functional in *E. coli*. Southern blot analysis indicated that the soybean AS is part of a small gene family. AS transcript was expressed in all tissues examined, but higher levels were seen in stem and root of light-grown tissue and leaves of dark-treated tissue.

Introduction

In many higher plants such as soybean, asparagine is an important nitrogen transport and storage amino acid during the processes of germination, nitrate assimilation, and nitrogen fixation [28, 40]. The synthesis of asparagine is mediated by the enzyme, asparagine synthetase (AS) (EC 6.3.5.4), which catalyzes the ATP-dependent transfer of the amide group of glutamine (or ammonia) to aspartate, producing asparagine and glutamate. AS has been isolated and partially purified from several plant sources: lupin [35, 36, 37], [15, 28], soybean [20, 42], maize [43] and alfalfa [44]. Plant AS has not been purified to homogeneity nor have the isoforms been separated or independently characterized. Two forms of AS have been described: a glutamine-dependent form [19, 22, 28, 36, 37] and an ammonia-dependent form [7]. The glutamine-dependent form is the most common form and is present in bacteria, plant and mammalian cells. This form is capable of catalyzing both reactions, although glutamine is the preferred nitrogen source. The ammonia-dependent form is found mainly in bacteria and is only capable of catalyzing the reaction in which ammonia is the nitrogen source. However, AS in maize roots can utilize ammonia effectively under excess ammonia [43].

AS is not characterized extensively at the biochemical level because of its extreme instability *in vitro* [20, 21, 36, 37]. To further compound the problem, partially purified AS preparations contain contaminating asparaginase activity [41] and heat stable inhibitors of AS [24], which make it difficult to assay AS activity *in vitro*. Therefore, purifying AS to homogeneity from plants is a formidable task.

The nucleotide sequence data reported will appear in the EMBL GenBank and DDBJ Nucleotide Sequence Databases under the accession number 77679 (AS1) and 77678 (AS2).

To circumvent some of these difficulties, we decided to clone the AS gene from soybean and functionally express it in *E. coli*. The overproduction of AS in *E. coli* would allow us to obtain large quantities of the protein for purification, antibody production and kinetic analysis.

With asparagine's pivotal role as a nitrogenous transport and storage compound in higher plants, we have focused on defining the biochemical and genetic mechanisms that regulate the expression of AS enzyme(s). This knowledge may enable us to determine how to direct more nitrogen in soybean towards economically important end products. In this study, we describe the isolation, characterization and expression of two cDNA clones encoding AS from soybean.

Materials and methods

Materials

All plant tissues were isolated from soybean (*Glycine max* L.) cv. Century. Tissues were harvested from seedlings germinated in vermiculite in the greenhouse. Dark-grown tissue was harvested from seedlings grown in vermiculite and was placed in boxes wrapped in dark plastic bags. The leaf tissue used as the source of mRNA for cDNA synthesis was grown on water-saturated paper towels in the green house [25].

Escherichia coli cells XL1Blue [6] were obtained from Stratagene. The asparagine synthetase auxotroph (ER) (*asnA31,asnB32,thi-1,relA1,spot1*) [7] was obtained from *E. coli* Genetic Stock Center [2].

Restriction enzymes, T_4 ligase, DNA and RNA markers, alkaline phosphatase from calf intestine, *Taq* DNA polymerase, and pUC18 were purchased from Gibco Bethesda Research Laboratories, Life Technologies (Gaithersburg, MD) or Boehringer Mannheim (Indianapolis, IN). Vectors pIBI30 and pIBI31 were from International Biotechnologies (New Haven, CT). Digoxigenin DNA labeling and detection system was purchased from Boehringer Mannheim (Indianapolis, IN). Oligonucleotides were synthesized on an Applied Biosystem's DNA synthesizer. Common chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Isolation of soybean AS cDNA clones

Degenerate oligonucleotide primers (AS5-1, 5′-GTN-AAT/CGGNGAA/GATC/T/ATAC/TAAC/TCAC/TG-AA/GG-3′; AS3-1, 5′-GG(N)GCC/TTTA/GTGG/TA-AA/GTA(N)AA/GA/GTA(N)CC-3′) were synthesized to resemble highly conserved regions of the AS gene of *Pisum sativum* [46] and man [1]. These primers were used in a polymerase chain reaction (PCR) [11] with soybean cDNA made from mRNA from light-grown cotyledon to amplify a portion of the AS gene. PCR cycling conditions were 94 °C for 3 min for initial denaturation followed by 94 ° for 1 min, 47 °C for 2 min, and 72 °C for 3 min (30 cycles) with a 7 min final extension. The amplification generated a 0.8 kb PCR product. A partial AS cDNA clone (SAS1, 5′ end, 1114 bp) was isolated from a λgt11 cDNA library constructed from mRNA extracted from 5-day old dark-grown cotyledons, using a 0.8 kb PCR product as a probe. Several clones were analyzed and contained partial cDNA fragments that were highly homologous to the 5′ end of both pea AS1 and AS2 DNA sequences. One clone SAS1 (5′ end, 1114 bp) was isolated, restriction sites were mapped, and the entire clone was sequenced.

Later, the 3′ end of SAS1 was isolated using the 3′ RACE (rapid amplification cDNA ends) procedure [16]. cDNA was synthesized from mRNA extracted from 2-day dark-treated leaves using the universal primers (618, CGCGGATCCTGCAGGTAC CTTTTTTTTTTT$(TTT)_3$TTTTTG; 619, CGCGGAT-CCTGCAGGTACCTTTTTTTTTTT$(TTT)_3$TTTTTC; 620, CGCGGATCCTGCAGGTACCTTTTTTTTTTT $(TTT)_3$TTTTTA) with restriction sites *Bam*HI, *Kpn*I, *Pst*I. One-tenth of the cDNA mixture was used in a PCR amplification reaction using a gene specific primer (665, CCGGAATTCCGGGTACATAG-GAACTGTCCATCATGAATTTCACTAC) containing an *Eco*RI site along with the adapter primer (4927, CGCGGATCCTGCAGGTACC) containing *Bam*HI, *Kpn*I and *Pst*I sites. The amplification generated a fragment of 1.3 kb. This 1.3 kb product was sequenced and compared to other AS genes.

The SAS1 probe was prepared by isolating a 1114 bp *Eco*RI fragment of the partial soybean cDNA clone by preparative gel electrophoresis [3] and radiolabeled with [α-^{32}P]dCTP using the random priming method of Feinberg and Vogelstein [12]. Filters were hybridized in 50% formamide, 1× Denhardt's solution [10], 6× SSC (1× SSC is 150 mM sodium citrate pH 7, 15 mM NaCl), 0.1% (w/v) sodium dodecyl sulfate (SDS), and 200 μg/ml denatured salmon sperm DNA at 42 °C for 18 h. The filters were washed twice in 0.1× SSC/0.1% (w/v) SDS at 42 °C for 30 min and exposed to X-ray film, XAR-5 for 3 days at −80 °C.

AS2 cDNA clones were selected from a λZAP II cDNA library that was previously constructed from *Glycine max* (cv. Century) mature leaf mRNA [25]. The phage were plated and screened according to the methods described by Maniatis *et al.* [30]. About 168 000 plaques were screened using radiolabeled SAS1 as a probe.

One of the clones, lambda SAS2, which hybridized to the 5′ end of SAS1, was chosen for further study. Phagemid DNA (pBluescript) containing the pSAS2 cDNA insert was isolated from lambda SAS2 through the excision process by co-infection with helper phage (M13KO7) [30]. Further isolation was performed using the alkaline lysate method [30]. The *Eco*RI insert of pSAS2 was digested with *Xba*I and the resulting fragments were subcloned into plasmid vector plBI31 for nucleotide sequencing. DNA sequencing analyses were performed in both directions on double stranded plasmid DNA using the dye terminator method [8] at Nucleic Acid Facility, lowa State University. SAS2 DNA sequence was analyzed using the University of Wisconsin GCG sequence analysis package running on the Vax 8250 system (Genetics Computer Group). Nucleotide and protein sequence comparisons were determined using the Needleman and Wunsch [34] algorithm (Gap). For multiple sequence comparisons, the method of Feng and Doolittle [14] (Pileup) was used. A dendrogram was constructed using the distance algorithm, Kimura method to determine evolutionary relationships between several AS amino acid sequences from different plants, human and *E. coli* [26].

DNA and RNA analysis

Genomic DNA was isolated from soybean leaves [32], and 10 μg of DNA was digested with restriction endonucleases *Bam*HI, *Eco*RI, *Hin*dIII, *Sst*I, and *Xba*I. The digested DNA was resolved by electrophoresis on a 1.0% agarose gel and transferred onto nylon membrane by capillary action [30]. The nylon membrane was hybridized with the 590 bp partial fragment (nucleotides 1359–1949) of pSAS2 which included the 3′ noncoding region. The partial fragment (nucleotides 1359–1949), 590 bp, of the SAS2 clone was labeled using the digoxigenin labeling kit (Boehringer-Mannheim Biochemicals) according to manufacturer's instructions. The nylon membrane was prehybridized in 5× SSC, 1% Genius blocking reagent (Boehringer-Mannheim Biochemicals), 0.1% (w/v) N-lauroyl sarcosine, 0.02% (w/v) SDS at 65 °C for 3 h. The digoxigenin-labeled probe was added to the prehybridization solution and hybridized at 65 °C for 18 h. The blot was washed two times at a stringency of 0.5× SSC/0.1% (w/v) SDS at 65 °. The final two washes were at room temperature using 1× SSC/0.1% SDS. Chemiluminescent detection was performed according to the instructions of Boehringer Mannheim Genius System User's Guide version 3.0.

For northern blot analysis, total RNA was isolated from different soybean tissues (roots, stems, light-grown cotyledons and leaves) 11 and 13 days (leaves only) after planting and from dark-adapted leaves (2 days) [30]. A 25 μg portion of total RNA from roots, stems and light-grown cotyledons and 4 μg of poly(A)$^+$ RNA from leaves were separated on a denaturating formaldehyde agarose gel and transferred to nylon membrane [30]. Total RNA blot was hybridized to digoxigenin-labeled AS2 probe (nucleotides 1359–1949; 590 bp) whereas the poly(A)$^+$ RNA blot was hybridized to ^{32}P-labeled AS2 probe (nucleotides 1173–1989; 816 bp) which both included the 3′ end of SAS2. The gels were stained with ethidium bromide to confirm that equal quantities of total RNA and poly(A)$^+$ RNA were loaded in each lane. The total RNA blot was hybridized in 5× SSC, 2% blocking solution, 50 mM NaH_2PO_4, 0.1% N-lauroylsarcosine, 7% SDS, 50% formamide at 42 °C for 18 h. For the poly(A)$^+$ blot, hybridization conditions were as follows: 1 M sodium phosphate pH 7.2, 20% SDS, 5% BSA, 0.5 M EDTA and 200 μg/ml of denatured salmon sperm DNA at 42 °C for 18 h. The final wash for the total RNA blot was at 42 °C with a stringency of 0.5× SSC/0.1% SDS and chemiluminescent detection was performed on total RNA blot according to the manufacturer's guidelines. For the poly(A)$^+$ blot, the final wash was 0.1× SSC/0.1% SDS at 55 °C and the blot was exposed to X-ray film for 5 days at −80 °C.

Construction of expression plasmid

The region corresponding to the open reading frame was amplified by polymerase chain reaction (PCR) [11]. One oligonucleotide, AS5-23 (5′-CCGGAATTCCGGATGTGTGGTATTCTTG-3′) contained the proposed glutamine-binding site and a 5′ *Eco*RI site extension for cloning. The inverse oligonucleotide AS5-24 (5′-CGCGGATCCGCGCTT-TGTCACATCTTTG-3′) corresponded to a region that was 3′ to the stop codon and contained a 5′ *Bam*HI site extension. These primers were designed such that the amplified product would be cloned in-frame with the

*lac*Z gene using the vector pUC18. The PCR reaction, 50 μl, contained 20 mM Tris-HCl pH 8.4, 50 mM KCl, 2.5 mM $MgCl_2$, 100 μg/ml BSA, 10 mM DTT, 200 μM each dATP, dCTP, dGTP and dTTP, 200 nM AS5-23, 200 nM AS5-24, 0.74 μg of plasmid DNA and 1.25 units of *Taq* polymerase. PCR cycling conditions were 94 °C for 3 min for initial denaturation followed by 94 °C for 45 s, 37 °C for 25 s and 72 °C for 3 min (30 cycles) with a 15 min final extension at 72 °C. The 1788 bp product was gel-isolated [3], digested with the restriction enzymes *Bam*HI and *Eco*RI, and cloned into *Bam*HI and *Eco*RI sites of plasmid pUC18 vector. The resulting plasmid pSAS2E was used to transform *E. coli* XL1Blue cells. Plasmid DNA was isolated from the positive colonies and digested with several restriction enzymes to determine whether any modifications had occurred.

Complementation studies

An *E. coli* AS auxotroph ER [7, 13]; *asnA31 asnB thi-1 relA1 spoT1*) was transformed with the plasmids, pUC18 (as control) or pSAS2E and plated on M9 plates containing 1 mM isopropylthio-β-D-galactoside (IPTG) and 100 μg/ml ampicillin (AMP). Several transformants of pSAS2E were selected and analyzed by restriction enzyme digestion and partially sequenced to determine whether any rearrangements had occurred. One clone SAS2E38 was used for further study. Cells containing pUC18 and pSAS2E38 were grown in liquid M9 medium overnight. The cultures were diluted 1 to 100 in fresh M9 medium containing either: (+) 100 μg/ml asparagine (+) 100 μg/ml ampicillin; (−) 100 μg/ml asparagine (+) 100 μg/ml ampicillin; (+) 100 μg/ml asparagine (+) 1 mM IPTG (+) 100 μg/ml ampicillin; (−) 100 μg/ml asparagine (+) 1 mM IPTG (+) 100 μg/ml ampicillin to determine the growth pattern of these cells containing plasmids pUC18 or pSAS238. The optical density was measured at 550 nm after 0, 2, 4, 6, 8, and 18 h of growth.

Results

Cloning and characterization of soybean AS2 cDNA clone

Two cDNA clones (SAS1 and SAS2) encoding different asparagine synthetase isoforms were isolated. SAS2 was isolated from a λZAP cDNA library of 168 000 plaques, which was derived from mRNA extracted from mature leaves. A partial AS cDNA clone (SAS1, 5′ end, 1114 bp) was isolated from a λgt11 cDNA library constructed from mRNA extracted from 5 day old dark-grown cotyledons, using a 0.8 kb PCR product as a probe. The mature leaf cDNA library was screened using the 5′ end of SAS1 (1114 bp) as a probe. Later, the 3′ end of SAS1 was isolated using the 3′ RACE procedure [16].

The nucleotide and deduced amino acid sequences of SAS1 and SAS2 cDNAs are shown in Fig. 1. The proposed initiation ATG codon is located at nucleotides 1 to 3 for both SAS1 and SAS2. A 5′ in-frame stop codon (TGA) for SAS1 and (TAA) for SAS2 are both located 12 nucleotides upstream of the proposed start codon. This indicates that the translation of this open reading frame could not have been initiated 5′ to the ATG start codon. The regions upstream of the ATG start sites are 150 nt (SAS1) and 154 nt (SAS2) in length which could represent a 5′-untranslated leader region. The two soybean AS cDNAs are divergent within the 3′ noncoding regions which have been shown for the two closely related pea AS1 and AS2 genes [46]. Within the 3′ noncoding region (SAS2) there is a polyadenylation signal from nucleotide 1923 to 1929 (AAUAAAA). The signal has high homology to the putative polyadenylation signal sequence of plants [23]. The open reading frames from the proposed initiating Met to the termination codons (TAG) and (TAA) encode a 579 amino acid peptide for SAS1 and a 581 amino acid peptide for SAS2 with calculated relative molecular weights of 65 182 and 65 608 Da, respectively.

The deduced amino acid sequences of soybean AS1 and AS2 were aligned with the deduced amino acid sequences of pea AS1 and AS2, asparagus, *Arabidopsis*, human AS, and *E. coli asnB* (Fig. 2), to determine regions that were conserved within these sequences. There are several regions of high homology shared with soybean AS1 and AS2, pea AS1 and AS2, asparagus, *Arabidopsis*, human AS and *E. coli asnB* proteins. In particular, one region that is highly conserved in the AS proteins is the proposed glutamine-binding site. The site consists of the amino acids methionine, cysteine, glycine, isoleucine (MCGI), which is perfectly conserved in soybean AS1 and AS2, pea AS1 and AS2 [46], asparagus [9], *Arabidopsis* [27], and human AS [1] proteins. In *E. coli* (*asnB*) [38], the glycine is replaced with a serine residue.

Another region that is highly conserved among the AS proteins and glutamine amidotransferases (*E. coli* and *Saccharomyces cerevisiae*) [31, 50], which uses

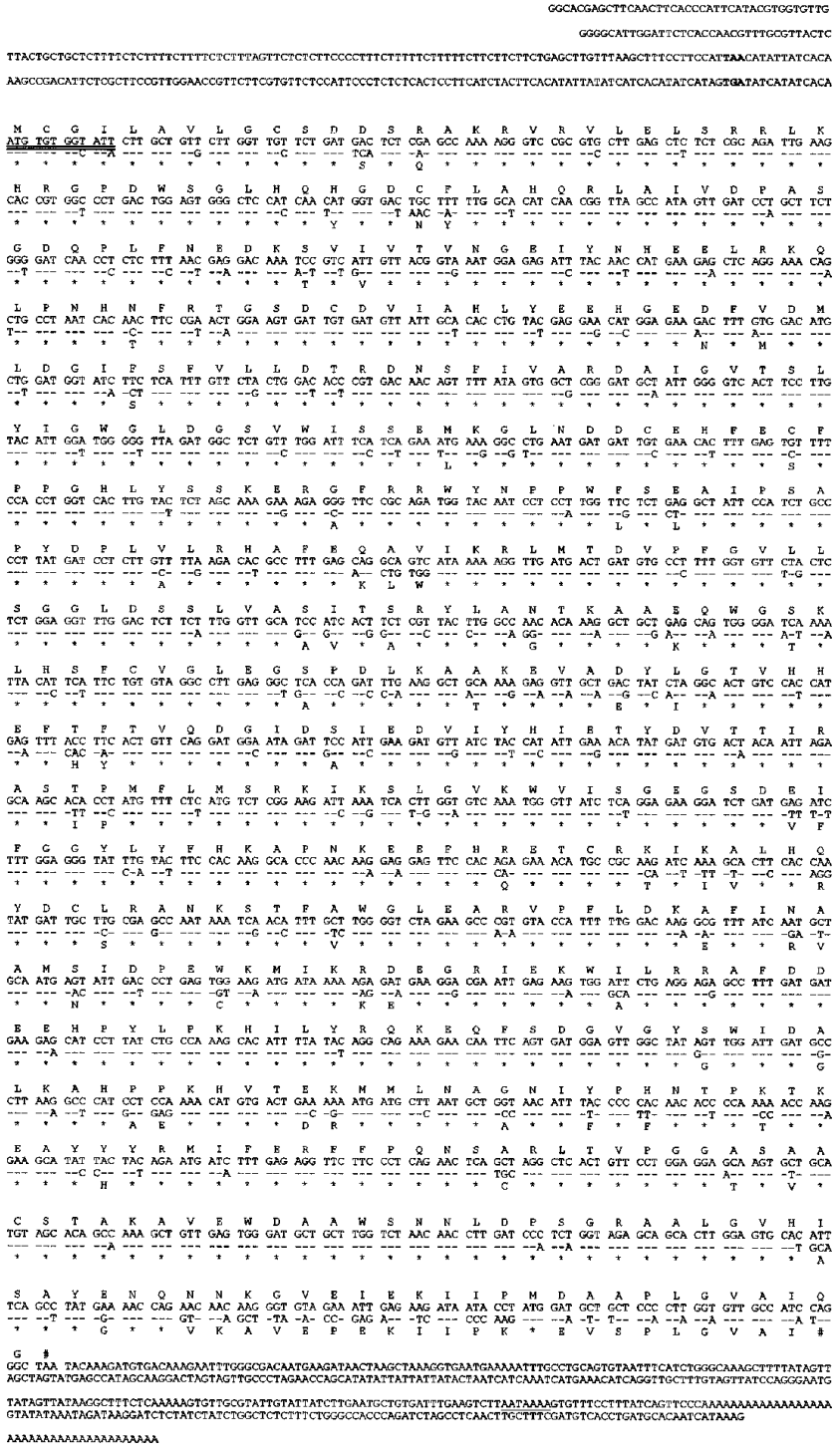

Figure 1. Nucleotide and deduced amino acid sequences of soybean AS1 and AS2 cDNA clones. SAS2 is the upper nucleotide sequence and the deduced amino acid sequence is denoted above in one-letter code. The nucleotide sequence of SAS1 is shown below where (−) represents identity of SAS1 to the upper sequence, SAS2. The deduced amino acid sequence of SAS1 is denoted below in one-letter code where (*) indicates identity with the upper amino acid sequence, SAS2. Proposed glutamine binding site is double-underlined. The translation termination codon is designated as #. The polyadenylation signal for SAS2 is underlined.

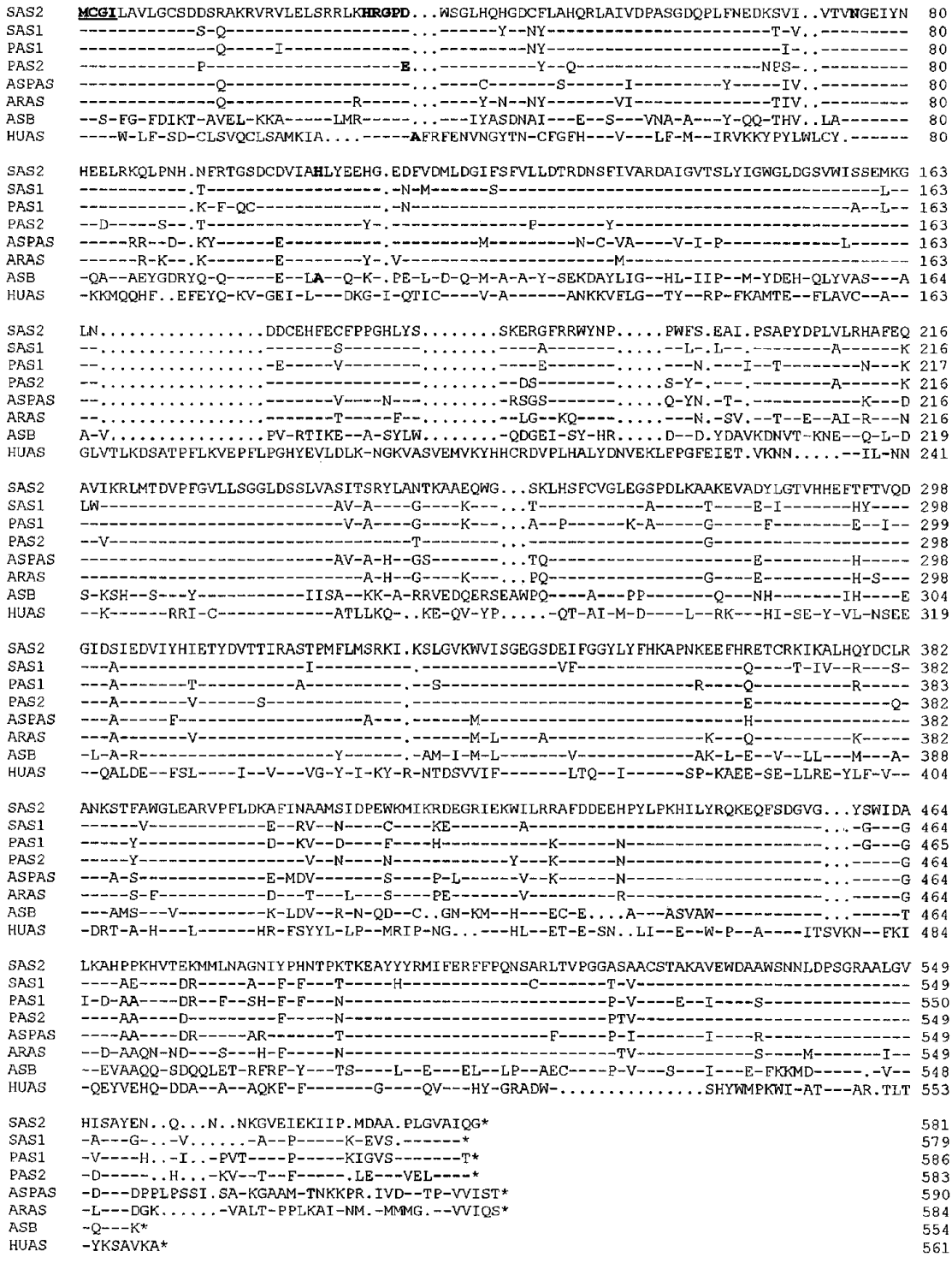

Figure 2. The amino acid alignment of several deduced AS peptides. The alignment was performed using the Genetics Computer Group PILEUP program of Feng and Doolittle [14]. The AS peptides used in the comparison alignment are: soybean AS (SAS1 and SAS2, this work), pea AS1 and AS2 (PAS1 and PAS2 [46]), asparagus AS (ASPAS [9]), arabidopsis (ARAS [27], *E. coli asnB* (ASB [38]), human AS (HUAS [1]). Dashes represent regions of identical amino acids whereas dots denote gaps which were used to maximize the homology of the AS peptides.

glutamine as a substrate like asparagine synthetase, is an internal region of 6 amino acid residues histidine, arginine, glycine, proline, aspartate and alanine. The proline residue was not conserved in the glutamine amidotransferases, whereas the alanine residue was not conserved in plant AS sequences (Fig. 2), but was conserved in the human AS sequence [1]. In pea AS2 the aspartate residue was replaced with glutamine.

A region referred to as the catalytic triad is conserved in amidotransferase and asparagine synthetase protein sequences (Fig. 2). The catalytic triad is involved in the glutamine amide transfer function of amidotransferases [31]. The triad is located at Cys-2, Asp-34, and His-104 in plant and *E. coli* AS sequences and Cys-2, Asp-34 and His-104 is replaced by alanine (Fig. 2). Arg-30 and Asp-74 which are Arg-31 and Asp-75 in soybean AS2, pea AS1 and pea AS2, *Arabidopsis*, asparagus, and human AS sequences are conserved. These residues have been reported to play functional roles in the glutamine-dependent activity of asparagine synthetase in *E. coli* [4]. Further, there are several other conserved regions that may represent binding sites for ATP and/or aspartate and/or glutamine (Fig. 2).

The predicted amino acid sequences of SAS1 and SAS2 showed significant similarity to several other AS sequences which are shown in Fig. 2. The SAS1 and SAS2 protein sequences showed the highest homologies to pea AS1 [46] (92%, 93%), pea AS2 [46] (91%, 96%), asparagus [9] (88%, 91%), *Arabidopsis* [27] (88%, 90.5%). The deduced amino acid sequences of SAS1 and SAS2 have less of a similarity to *E. coli asnB* [38] (70.5%, 72.5%) and human AS [1] (61%, 63%). The amino acid sequences of soybean, pea, asparagus, *Arabidopsis*, human, and *E. coli asnB* showed no significant similarity to the *E. coli asnA* amino acid sequence [33]. The polypeptides encoded by SAS1 and SAS2 cDNAs share a homology of 91.5% at the amino acid level. Further, similarities and identities at the amino acid level along with the dendrogram (Fig. 3) indicate that the human, and *E. coli asnB* AS proteins are evolutionary more divergent from the soybean, pea, asparagus, and *Arabidopsis* AS proteins. Also, SAS2 is more closely related to pea AS2 than to pea AS1.

Genomic DNA was digested with several restriction enzymes and hybridized to a portion (nucleotides 1359–1949; 590 bp, 3′ end) of the SAS2 gene fragment. Several fragments of genomic DNA hybridized to the SAS2 DNA probe: 2 fragments with *Bam*HI, 5 fragments with *Eco*RI, 5 fragments for *Hin*dIII, 3 fragments for *Sst*I, and 4 fragments for *Xba*I (Fig. 4).

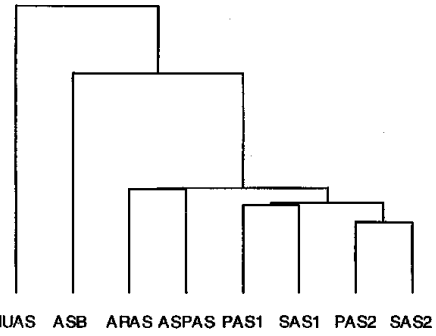

Figure 3. In this dendrogram, the Kimura protein distances algorithm was used [26]. The AS peptides used in the comparison alignment are: soybean AS (SAS1 and SAS2, this work), pea AS1 and AS2 (PAS1 and PAS2 [46]), asparagus AS (ASPAS [9], arabidopsis (ARAS [27]), *E. coli asnB* (ASB [38], human AS (HUAS [1]).

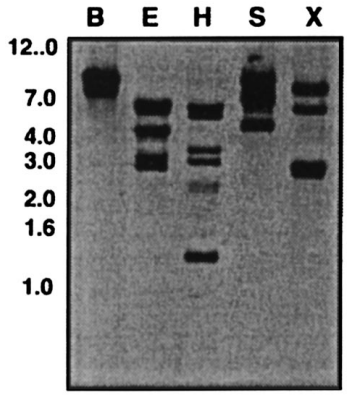

Figure 4. Southern blot analysis: soybean genomic DNA was digested with restriction enzymes *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), *Sst*I (S), *Xba*I (X), separated by agarose gel electrophoresis, transferred to nylon membrane and hybridized to a 590 bp (nucleotides 1359–1949) probe of SAS2 which contains the 3′ noncoding region.

To determine if the level of AS expression varied in different soybean tissues, RNA was isolated from soybean seedlings grown under normal light for 11 days. Northern analysis revealed a 2.2 kb AS transcript was expressed highly in root and stem tissue whereas lower levels were detected in light-grown cotyledon tissue (Fig. 5A). Further, to establish whether the AS transcript was induced during dark treatment, RNA was isolated from leaves grown under normal light for 13 days and leaves grown under normal light for 11 days and moved to the dark for 2 days (Fig. 5B). The AS transcript was more abundant in leaves shifted to the dark for 2 days than in leaves of plants grown in the light.

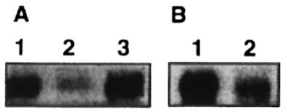

Figure 5. A. Northern blot analysis of total RNA isolated from several soybean tissues 11 days after planting, separated on a denaturating formaldehyde agarose gel, transferred to nylon membrane, and hybridized to a 590 bp probe (nucleotides 1359–1949). Total RNA (25 μg) was isolated from root (lane 1); light-grown cotyledon (lane 2) and stem (lane 3) grown in continuous light for 11 days. The AS probe which represented the 3′ end of the gene was used to detect a 2.2 kb AS transcript in 11-day root, light-grown cotyledon and stem tissue. B. Analysis of soybean AS mRNA levels in leaves of dark adapted plants compared to light-grown soybean seedlings. Poly(A)$^+$ RNA (4 μg) was isolated from leaves of soybean seedlings grown in continuous light for 11 days and transferred to the dark for 2 days (lane 1) and grown in continuous light for 13 days (lane 2). The AS probe (816 bp) which represented the 3′ end of AS detected a 2.2 kb AS transcript in light- and dark-treated leaves but AS levels were markedly increased in dark-treated leaf tissue.

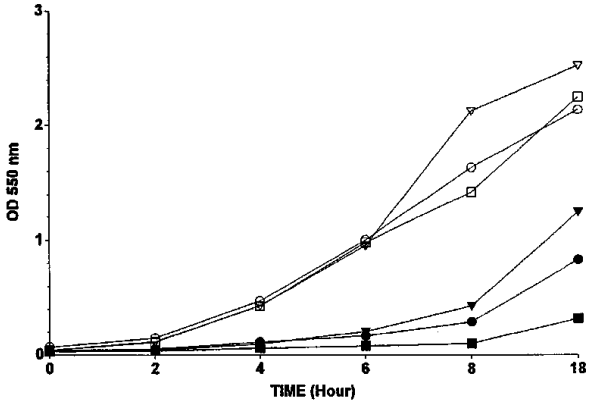

Figure 6. Complementation of an *E. coli* AS mutant (ER). Plasmids pUC18 (control) and pSAS2E were transformed into the AS mutant ER. Cultures were grown in M9 media in the presence and absence of 100 μg/ml asparagine and 1 mM IPTG. Growth was monitored at 550 nm at 0, 2, 4, 6, 8 and 18 h. ● ER/pUC18 (+) Asn; ■ ER/pUC18, (−) Asn; ▼ ER/pUC18, (+) Asn + 1 mM IPTG; ○ ER/pSAS2E, (+) Asn; □ ER/pSAS2E, (−) Asn; ▽ ER/pSAS2E, (−) Asn + 1 mM IPTG.

Complementation and expression of pSAS2E E. coli

The coding region of pSAS2 cDNA corresponding to the putative mature protein was placed in-frame in pUC18 and expressed in an *E. coli* auxotroph ER (*asnA, asnB, thi-1, relA, spoT1*) lacking AS activity [7, 13] (Fig. 5). *E. coli* ER transformed with pUC18 grew well in the presence of asparagine (100 μg/ml), but grew poorly in the absence of asparagine. On solid medium (M9 agar) without asparagine no growth was observed. Conversely, the *E. coli* ER transformed with pSAS2E grew extremely well in the absence of asparagine. Also, growth on solid medium was similar to what was observed in liquid culture. Complementation with pSAS2E occurred in the presence and absence of 1 mM IPTG. It appears that the expression of the AS gene was not tightly regulated by the *lacZ* promoter.

Discussion

In plants, the enzyme asparagine synthetase has not been characterized extensively at the biochemical level because of its instability *in vitro*. In efforts to study this enzyme in soybean, we decided to isolate the AS gene from soybean and to functionally express this gene in *E. coli*.

The soybean AS cDNA clones, SAS1 and SAS2 contain a large open reading frame of 1737 nt and 1743 nt respectively. The presence of an in-frame termination codon [TGA (SAS1), TAA (SAS2)], 12 nucleotides upstream of the putative ATG indicates that both SAS1 and SAS2 represent the full coding regions. A putative initiator methionine (ATG) is located at the 5′ end of the SAS1 and SAS2 cDNAs and the predicted proteins sizes are 579 and 581 amino acids with a calculated molecular mass of 65.1 kDa and 65.6 kDa respectively. The molecular masses of SAS1 and SAS2 are similar to the predicted molecular mass of several plant AS proteins: pea AS1, 66.3 kDa and AS2, 65.6 kDa [46]; asparagus, 66.5 kDa [7]; *Arabidopsis*, 65.5 kDa [27]; and of the isolated AS proteins of man, 64 kDa [39, 47], bovine pancreas, 60 kDa [29]; rat liver, 60 kDa [18] and rat testes, 62 kDa [17].

In pea, two distinct AS genes have been identified, AS1 and AS2 [46]. These genes are very similar at the 5′ end and divergent at the 3′ noncoding region. The two soybean AS cDNAs are highly homologous to each other at the nucleotide level within the coding regions (85%) and the 3′ noncoding regions of 220 nucleotides have little homology (Figs. 1 and 2). There is a 91.5% similarity and 87% identity between the two soybean AS at the amino acid level. SAS2 has a higher identity to pea AS2 than to pea AS1 and the predicted protein size of SAS2 is identical to the predicted size of pea AS2 suggesting that SAS2 maybe the soybean homologue of pea AS2 (Figs. 2 and 3). Analysis of the sequences and the dendrogram does not clearly indicate that SAS1 is the soybean homologue of pea AS1 (Figs. 2 and 3).

Several AS protein sequences and the glutamine amidotransferases of *E. coli* and *S. cerevisiae* con-

tain a highly conserved domain which is the proposed glutamine binding site. Van Hack and Schuster [49] demonstrated that replacement of the N-terminal cysteine of the human AS protein with an alanine residue resulted in the loss of glutamine-dependent AS activity, but the ammonia-dependent activity remained. Also, Cys-1-Ala and Cys-1-Ser mutants possessed no glutaminase activity [49]. Brears *et al.* [5] reported similar results in transgenic plants containing a deletion mutant of the AS1 gene which lacked Cys-2, Ile-3 and Gly-3 fused to the 35S promoter. These plants expressed the ammonia-dependent activity [5]. These results indicate that the cysteine residue along with the isoleucine and glycine residues are very important for glutamine-dependent AS activity. Additional residues such as Arg-30, and Asp-74 have been implicated in providing AS with Glu-dependent activity [4]. Using the *E. coli* ASB, Boehlein *et al.* [4] have reported that Arg-30 appears to mediate interaction between the synthetase and glutamine amide transfer regions on the enzyme whereas Asp-74 is involved in catalyzing the reaction for the transfer of nitrogen from glutamine. Furthermore, the conservation of the proposed glutamine binding site along with Arg-31 and Asp-75 in SAS1 and SAS2 may indicate that SAS1 and SAS2 cDNA encodes the glutamine-dependent form of AS in soybean.

In addition, there are other regions of amino acid conservation. SAS1 and SAS2 contain the conserved amino acids His-30, Arg-31, Gly-32, Pro-33 and Asp-34 which is replaced by glutamate in pea AS2. Among the glutamine amidotransferase sequences, conservation occurs only with the histidine, arginine, glycine, aspartate residues [49]. Further site-directed mutagenesis of Asp-29 and His-101 of glutamine phosphoribosyl-pyrophosphate amidotransferases resulted in the loss of glutamine but not ammonia-dependent activity [31] which may have similar effects on the asparagine synthetases. The amino acids aspartate-29 and His-101 of the glutamine phosphoribosyl-pyrophosphate amidotransferase and human AS sequences which correspond to Asp-34 and His-104 of soybean AS1 and AS2, pea AS1 and AS2, *Arabidopsis* and asparagus AS sequences are conserved. These regions may also function together in creating a domain for glutamine binding.

In eukaryotes, proteins that are nuclear encoded and transported to specific organelles possess transit polypeptide sequences. Based on the location of the putative start codon, SAS1 and SAS2 does not contain a transit polypeptide sequence and appears to be the cytosolic form of AS. Presently, only genes encoding cytosolic AS have been isolated from pea [46], asparagus [9], *Arabidopsis* [27]. There is no evidence that soybean contains other distinct organellar AS genes.

Our genomic DNA analysis data revealed that several fragments of genomic DNA hybridized to the 3′ end of SAS2 DNA probe for all of the enzymes except for *Bam*HI in which 2 fragments were generated. These data indicate that SAS2 is part of a small gene family.

Northern blot analysis revealed higher levels of AS mRNA in root and stem tissue as compared to light-grown cotyledon tissue when plants were grown in continuous light for 11 days. Previous data, Tsai and Coruzzi [45], indicated that AS1 and AS2 mRNA levels were negatively regulated by light in leaf and stem tissue whereas AS2 mRNA accumulated independently of light in the roots of plants grown in continuous light for 28 days. Our data suggest that in younger soybean seedlings large quantities of AS is required in the roots and stems for the synthesis of asparagine as compared to older seedlings which may explain the difference of AS mRNA expression in stem tissue of soybean and pea seedlings. When seedlings were grown in continuous light for 11 days and then transferred to the dark for 2 days, AS mRNA levels accumulated to higher levels in leaf tissue as compared to AS mRNA from leaves grown in continuous light for 13 days. In general, AS mRNA accumulates to high levels in leaves of dark treated soybean seedlings and light represses this effect as has been reported for *Pisum sativum* [45, 46].

The open reading frame of SAS2, representing the putative mature AS protein, was expressed in an AS *E. coli* auxotroph. The auxotroph ER contains a mutation in both the *asnA* and *asnB* genes of *E. coli*, thus the bacterial AS enzymes are not functional. Therefore, asparagine is required for growth of the auxotroph in M9 medium. After transforming ER with the soybean AS construct, a normal growth pattern resulted in M9 medium without the asparagine supplement. Complementation of the AS auxotrophy in *E. coli* was not dependent on the presence of IPTG and was not temperature-dependent because growth occurred at 37 °C. In contrast, complementation of the human AS gene in *E. coli* was temperature-dependent because no growth resulted at 37 °C, only at 30 °C. Another problem that was encountered was the insolubility of the recombinant human AS gene which seemed to be a result of temperature-sensitivity of the human protein [48]. These problems were not encountered with the expression of the soybean gene in *E. coli*. Our data

suggest that the soybean AS gene complemented the AS auxotroph in *E. coli* and expression of the protein appears not to be temperature-dependent at 37 °C. Future studies will include isolating the soybean AS protein from *E. coli* extracts, producing antibodies to purified protein and examining tissue specific expression of AS protein.

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